The trypanosome alternative oxidase

Menzies, Stefanie K.; Tulloch, Lindsay B.; Florence, Gordon J.; Smith, Terry K.

Published in:
Parasitology

DOI:
10.1017/S0031182016002109

Publication date:
2018

Document Version
Peer reviewed version

Full title: THE TRYPANOSOME ALTERNATIVE OXIDASE: A POTENTIAL DRUG TARGET?

Authors: Stefanie K Menzies, Lindsay B Tulloch, Gordon J Florence and Terry K Smith

Postal Address: Biomedical Sciences Research Complex, University of St Andrews, St Andrews, Fife, Scotland KY16 9ST

Running Title: TAO: A Potential Drug Target?

Corresponding author: Terry K Smith, Biomedical Sciences Research Complex, University of St Andrews, St Andrews, Fife, Scotland KY16 9ST 01334 463412

tks1@st-andrews.ac.uk

This article has been published in a revised form in *Parasitology* [http://dx.doi.org/10.1017/S0031182016002109].

Summary

New drugs against *Trypanosoma brucei,* the causative agent of Human African Trypanosomiasis, are urgently needed to replace the highly toxic and largely ineffective therapies currently used. The trypanosome alternative oxidase (TAO) is an essential and unique mitochondrial protein in these parasites and is absent from mammalian mitochondria, making it an attractive drug target. The structure and function of the protein are now well characterized, with several inhibitors reported in the literature which show potential as clinical drug candidates. In this review we provide an update on the functional activity and structural aspects of TAO. We then discuss TAO inhibitors reported to date, problems encountered with *in vivo* testing of these compounds, and discuss the future of TAO as a therapeutic target.

Key Words: Trypanosome Alternative Oxidase, Drug Discovery, Chemotherapy, Human African Trypanosomiasis, Sleeping Sickness, Trypanosoma brucei

THE TRYPANOSOME ALTERNATIVE OXIDASE: A POTENTIAL DRUG TARGET?

INTRODUCTION

Up to 70 million people in sub-Saharan Africa are at risk of contracting Human African Trypanosomiasis (HAT) (Simarro *et al*. 2012), also known as African Sleeping Sickness, caused by the kinetoplastid parasite *Trypanosoma brucei.* Two subspecies of the parasite cause disease in humans; *T. brucei gambiense* in West Africa and *T. brucei rhodesiense* in East Africa, both of which are spread by the tsetse fly. Both forms are fatal if untreated and are estimated to cause up to 20,000 cases of HAT per year (World Health Organization 2013). *T. brucei* evades the mammalian host immune system by changing their major surface coat proteins, known as variant surface glycoproteins (VSG), prior to each wave of host antibodies raised against the previous VSG type. Due to this sophisticated immune evasion technique known as antigenic variation, a vaccine against the disease is unlikely in the near future. Drugs currently in clinical use are associated with severe adverse effects, difficult administration, and increasing concerns regarding drug resistance. Therefore, new drugs are urgently required (Lüscher *et al.* 2007). The drugs indicated for treatment of the disease (Figure 1) depend upon the subspecies of parasite and stage of the disease.

Early stage *T. b. gambiense* is treated with pentamidine, a diamidine hypothesized to act as a trypanocidal agent through several mechanisms, including disruption of the nucleus, kinetoplast and mitochondrial membrane potential (Baker *et al.* 2013). Late stage *T. b. gambiense* is treated with a combinational therapy of nifurtimox and eflornithine. Eflornithine is the only drug for HAT with a defined target, the ornithine

decarboxylase, but the drug has poor potency against *T. brucei* and combination therapy is required to prevent drug resistance acquired by loss of the drug uptake transporter (Barrett & Croft 2012). Suramin is recommended only for early stage *T. b. rhodesiense* due to its inability to penetrate the blood brain barrier. Although the mechanism of uptake by the parasites is known, the trypanocidal mode of action still remains to be determined (Barrett & Croft 2012, Zoltner *et al.* 2016). The arsenical-based drug melarsoprol is recommended for late stage *T. b. rhodesiense* due to its ability to cross the blood brain barrier, however this property creates the often fatal adverse effect of encephalopathy in up to 10% of patients treated with the drug (Kuepfer *et al.* 2012).

Differences in the biochemical processes between mammalian and trypanosomatid mitochondria make the mitochondrion an attractive drug target. One main difference between *T. brucei* and mammalian mitochondrial respiration is the presence of the trypanosome alternative oxidase (TAO), an essential non-cytochrome terminal oxidase which has been extensively characterized as a drug target. This review will summarize the structure and function of TAO and discuss the current progress towards the development of inhibitors against this protein.

STRUCTURE AND FUNCTION OF THE TRYPANOSOME ALTERNATIVE OXIDASE

Function

In 1960, Grant and Sargent first described the glycerol-3-phosphate oxidase (GPO) system as a cyanide-insensitive, oxygen-dependent mechanism of respiration in *Trypanosoma brucei rhodesiense* (Grant & Sargent 1960). The GPO system consists of two enzymes; a mitochondrial FAD^+-dependent glycerol-3-phosphate

dehydrogenase (mG3PDH) and a terminal oxidase they termed the glycerol-3-phosphate oxidase. Clarkson et al (Clarkson *et al.* 1989) proved that ubiquinol links the dehydrogenase and oxidase of the GPO system by acting as an electron carrier, and proposed that glycerol-3-phosphate oxidase was similar to the plant alternative oxidase (AOX) and therefore should be renamed the trypanosome alternative oxidase (TAO). The GPO system is responsible for the cyanide-insensitive oxygen-dependent respiration in bloodstream form *T. brucei,* where the GPO shuttle facilitates the reoxidation of NADH to NAD^+ required for glycolysis. As shown in Figure 2, the mG3PDH oxidizes glycerol-3-phosphate (Gly-3-P) to dihydroxyacetone phosphate (DHAP), during which four electrons are transferred to ubiquinol. The electrons from ubiquinol are subsequently oxidized by TAO to convert dioxygen into water. Alternative oxidases are found across a broad range of organisms, including plants, nematodes, algae, yeast and *T. brucei*, but, curiously, are not known to be present in the other human-infective trypanosomatids such as *T. cruzi* or *Leishmania* spp.

TAO was first identified in *Trypanosoma brucei* by Chaudhuri et al (Chaudhuri *et al.* 1995) using antibodies against the alternative oxidase from *Sauromatum guttatum*, which detected a 33 kDa protein in the parasite's mitochondria. This 33 kDa protein was subsequently purified from bloodstream form *T. brucei* mitochondria and confirmed to have ubiquinol oxidase activity. Chaudhuri et al (Chaudhuri *et al.* 1998) found that bloodstream form *T. brucei* express TAO 100-fold more than procyclic form, which is believed to be due to the ability of procyclic forms to express Complexes III and IV for ATP production via oxidative phosphorylation. Using areas of high conservation in plant alternative oxidases, primers were designed to amplify TAO from *T. brucei* gDNA (Chaudhuri & Hill 1996). This enabled the identification of

the single copy TAO gene (Tb927.10.7090) and subsequent cloning of TAO for recombinant expression in *Escherichia coli* (Chaudhuri & Hill 1996). Recombinant TAO (rTAO) was subsequently used to determine the functional activity, kinetics and inhibitors of the enzyme. Due to the endogenous ubiquinol oxidase activity of *E. coli* by the cytochrome bo and bd complexes, it was necessary to perform these investigations using hemA mutant *E.* coli, which are unable to synthesize the heme necessary for cytochrome assembly. The ability of rTAO to restore respiration in these cells showed the ability of TAO to function as a cyanide-insensitive terminal oxidase. Research of rTAO by the Kita group established protocols for the overproduction, solubilization and purification of rTAO for use in kinetic, structural and inhibitor studies (Fukai *et al*. 1999; Fukai et al. 2003; Nihei *et al*. 2003; Yabu *et al.* 2003).

TAO has been implicated in several other cellular activities, such as protection against reactive oxygen species and regulation of surface protein expression. A role of AOX in photosynthetic plants is the rapid turnover of NADPH to protect the photosynthetic machinery from radicals. It is possible that TAO has a related function in *T. brucei*, to protect the rapidly metabolizing cells from damaging radicals. The inhibition of TAO has been shown to induce oxidative damage to proteins and increase production of reactive oxygen species (Fang & Beattie 2003). Similarly, inhibition of the electron transport chain and exposure to hydrogen peroxide causes an upregulation in the expression of TAO (Fang & Beattie 2003). This protection against oxidative damage may explain the ability of TAO to inhibit drug-induced programmed cell death-like phenomena in *T. brucei* (Tsuda *et al*. 2006). Vassella et al (Vassella *et al.* 2004) reported the effects of TAO inhibition on the expression levels of the procyclin GPEET, a cell surface protein found in procyclic form *T.*

brucei. In the presence of the TAO inhibitor salicylhydroxamic acid (SHAM), GPEET levels were heavily reduced, leading the authors to hypothesize that the level of GPEET expression may be linked to the activity levels of TAO. Later studies showed that the expression of TAO influences the expression of GPEET, where a downregulation of both proteins may be important in the adaptation of the parasite to survive within the tsetse fly midgut (Walker *et al.* 2005).

Structure

Several structures of the alternative oxidase were proposed (Andersson & Nordlund 1999; Berthold *et al.* 2000) prior to the publication of the crystal structure. Initially, hydropathy plots suggested the alternative oxidases contain two conserved transmembrane regions, however later studies by Andersson and Nordlund (Andersson & Nordlund 1999) suggested alternative oxidases are not transmembrane proteins, but rather interfacial inner membrane proteins. This was confirmed with the solving of the crystal structure of TAO (Shiba *et al.* 2013) which is devoid of any transmembrane domains, and instead has a hydrophobic face to partially bury the protein into the membrane (Figure 3). The recent publication of the crystal structure (Shiba *et al.* 2013) should help in the design of improved TAO inhibitors. Sequence analysis of *T. b. brucei, T. b. gambiense* and *T. b. rhodesiense* showed that the amino acid sequence of TAO is identical in all three species, and therefore studies on TAO inhibitors and its co-structures can be directly applied from the common laboratory model *T. b. brucei* to the human disease-causing subspecies (Nakamura *et al*. 2010).

Early studies on plant alternative oxidases revealed that they were inhibited by metal chelators (Schonbaum *et al.* 1971), and subsequent investigations using electron

paramagnetic resonance (EPR) (Berthold *et al.* 2002; Moore *et al*. 2008) and inductively coupled plasma-mass spectrometry (ICP-MS) (Kido *et al.* 2010) showed that the alternative oxidases contained a non-heme diiron catalytic core that was essential for catalytic activity and released during enzyme inactivation. In the crystal structure in its oxidized state, the two Fe (III) ions are coordinated in a distorted square pyramidal geometry to four glutamate residues and a hydroxo-bridge (Figure 4). Two conserved histidines located nearby are also likely to be involved in Fe-coordination in the reduced state, as determined through Fourier transform infrared spectroscopy (FTIR) investigations (Marechal *et al.* 2009). Together, the two histidines and two glutamates form part of the two ExxH iron-binding motifs which are common to all AOX proteins and are required for activity (Chaudhuri *et al.* 1998; Ajayi *et al.* 2002).

Mechanism of Catalysis

The structure of TAO in complex with ubiquinol has not yet been solved so hypotheses regarding ubiquinol-binding have been made based upon the structure of TAO complexed with ascofuranone analog AF2779OH. Superposition of ubiquinol over AF2779OH indicates that during catalysis a ubiquinol molecule is highly likely to occupy the same position. The molecules gain entry to the diiron active site through a relatively short (~10 Å) hydrophobic channel from the membrane-bound side of TAO (Figure 4). In this position the aromatic head of ubiquinol is less then 4.4 Å from the diiron core and is capable of forming hydrogen bonds with Arg118, Cys119 and Tyr220, all of which may be involved directly in catalysis rather than purely substrate binding.

A mechanism of catalytic activity has been proposed (Moore *et al.* 2013 and Figure 5), which begins with the diiron core in a reduced state (i.e. as Fe(II/II) bridged by a hydroxide). Upon binding of molecular oxygen to the Fe(II/II) diiron core (Figure 5A), one iron passes an electron to an oxygen atom, forming a superoxo intermediate comprising an oxygen radical joined to an Fe(II/III) core. The oxygen radical immediately abstracts a hydrogen atom (proton plus electron) from ubiquinol, yielding a ubisemiquinone and a hydroperoxo intermediate (Figure 5B). The unstable intermediate then undergoes a rearrangement whereby the hydroperoxo loses its proton and electron to the hydroperoxide bridge, which is then released as water (Figure 5C). The Fe(II/III) core then gains an interaction with one of the histidines as determined in FTIR experiments (Marechal *et al.* 2009) and the second atom of the dioxygen forming a peroxodiiron. Homolytic cleavage of the O-O bond (Figure 5D) yields an oxodiiron core, and one of the oxygens abstracts a hydrogen atom (proton plus electron) from Tyr220 generating a tyrosyl radical, as observed by Marechal et al (Marechal *et al.* 2009). The tyrosyl can then pick up an electron and proton from the ubisemiquinone, either directly or via Cys119, releasing ubiquinone and returning Tyr220 to its resting state. Moore's model suggests that ubiquinol in a second channel can then provide two electrons and protons to release a second water and reduce the diiron core back to its original Fe(II/II) state bridged by a hydroxide ion through an unknown mechanism (Figure 5E). However, the second ubiquinol channel may not be needed as the release of ubiquinone creates the space for the binding of a second ubiquinol in the same channel in a ping-pong binding fashion. Furthermore, the mechanism of electron and proton transfer could proceed through a similar route as for the first ubiquinol.

INHIBITORS OF THE TRYPANOSOME ALTERNATIVE OXIDASE

The effectiveness of TAO inhibition to kill *T. brucei* has been well debated, with conflicting historical reports in the literature as to whether the inhibition of the GPO system alone is sufficient to kill the cells. As shown in Figure 2, bloodstream form *T. brucei* rely solely on glycolysis for ATP production, as opposed to the ATP-producing oxidative phosphorylation used by procyclic forms. In the presence of TAO inhibitors the oxidation of Gly-3-P to DHAP is blocked, causing an accumulation of Gly-3-P in the glycosome, which is converted to glycerol by the ATP-producing glycerol kinase (Yabu *et al.* 2006). This allows the recycling of glycosomal NAD^+/NADH necessary to continue glycolysis anaerobically.

Early reports of *in vivo* testing of TAO inhibitors suggested that although the compounds were able to inhibit the protein *in vitro,* this action alone was not sufficient to clear an infection when tested in animal models, due to anaerobic ATP production by the trypanosomes (Clarkson & Brohn 1976; Grady *et al*. 1993; Yabu *et al*. 1998). It was believed that in order to cause cell death the anaerobic production of ATP also needed to inhibited with the co-administration of glycerol. However, later investigations showed that bloodstream *T. brucei* exposed to TAO inhibitors alone are unable to survive for more than 24 hours using only anaerobic respiration (Helfert *et al*. 2001). Furthermore, subsequent studies of a TAO inhibitor with an optimized dosing regimen but in the absence of glycerol, showed that TAO inhibition alone is sufficient to clear an infection *in vivo* (Yabu *et al.* 2003), indicating that inhibition of TAO is indeed a valid drug target.

There are few compounds that have been shown to be inhibitors of TAO. These compounds (Figure 6) all show structural similarity to the TAO substrate ubiquinol and are thought to act as competitive inhibitors, by binding to the ubiquinol binding

site.

Salicylhydroxamic Acid

The first compounds to be investigated as TAO inhibitors were the aromatic hydroxamates, such a salicylhydroxamic acid (SHAM) (Figure 6). SHAM was known to be a potent inhibitor of the alternative oxidase in plants prior to the discovery of the GPO system in trypanosomes, hence the compound was investigated as a potential inhibitor of TAO. It is thought that hydroxamic acids compete with ubiquinol for binding to TAO, and thus the compounds prevent the translocation of electrons from ubiquinol to oxygen (Pollakis *et al*. 1995). SHAM was found to have moderate (EC_{50} = 15 μM) activity against *T. brucei in vitro* and was shown to specifically inhibit all TAO activity at 1 mM (Opperdoes *et al*. 1976), although only a little effect was seen on ATP production. However, when the trypanocidal effect of SHAM was investigated *in vivo,* the compound was unable to clear an infection and was only shown to be trypanocidal when co-administered with glycerol (Clarkson & Brohn 1976).

SHAM is a poor clinical candidate, due to its low solubility in water (Nihei *et al.* 2002), which impairs the compounds from crossing the blood brain barrier, a critical characteristic required for drugs to effectively treat HAT. Numerous attempts were made to improve the potency of hydroxamic acids against TAO, but were unable to match the potency of SHAM when tested *in vivo* (Grady *et al*. 1993). Recently this issue has been revisited, Ott et al (Ott *et al.* 2006) developed novel SHAM analogs to improve its potency and solubility. SHAM analogs such as ACD16 (Figure 6) were designed to include a prenyl side chain, as found in the TAO substrate ubiquinol, and a carbohydrate group to improve solubility, whilst keeping the 2-hydroxybenzoic acid

found in SHAM which is essential for TAO inhibition. These modifications lead to the development of three compounds with up to five-fold greater potency than SHAM against rTAO, however *in vitro* testing against *T. b. brucei* growth and respiration revealed none of the modified compounds were more potent than SHAM. There have been no subsequent reports on SHAM as a TAO inhibitor, although recent reports on the efficacy of TAO inhibitors without glycerol (Yabu et al. 2003) may renew interest in attempts to improve upon this compound.

3,4-Dihydroxybenzoic Acid

3,4-dihydroxybenzoic acids (Figure 6) were synthesized and tested as alternative inhibitors of TAO, and displayed higher inhibitory activity than SHAM when tested *in vitro,* but this high potency was lost when the compounds were tested *in vivo* (Grady *et al.* 1993). To improve the bioavailability of the compounds, a series of N-*n*-alkyl-3,4-dihydroxybenzamides were synthesized to increase solubility and decrease hydrolysis by serum esterases (Grady *et al.* 1993). Structure activity relationships of this series of compounds showed increasing potency and decreasing solubility as the length of the alkyl substituent increases. From this, *N*-*n*-butyl-3,4-dihydroxybenzamide progressed to *in vivo* studies, and was found to effectively cure mice, but only when administered in conjunction with high doses of glycerol (450 mg/kg drug with 15 g/kg glycerol). Similar to SHAM, the high amount of glycerol necessary for a trypanocidal effect of *N*-*n*-butyl-3,4-dihydroxybenzamide rendered the compound unfavourable as a clinical drug candidate, and no work has been undertaken to identify if an optimized dosing regimen might clear infection *in vivo* without glycerol.

Ascofuranone

Ascofuranone (Figure 6), is a biologically active natural product isolated from the fungus *Ascochyta viciae.* Minagawa et al first showed that ascofuranone is a potent inhibitor of mitochondrial respiration of *T. b. brucei,* specifically the glucose- and glycerol-3-phosphate-dependent respiration (Minagawa *et al*. 1997). Despite its high potency against TAO, ascofuranone was initially found to only be trypanocidal in the presence of glycerol, similar to the other TAO inhibitors. The minimum inhibitory concentration of ascofuranone alone was 250 μM, whereas in the presence of 4 mM glycerol potency was improved several thousand-fold to 30 nM (Minagawa *et al.* 1997). Initially *in vivo* testing using mouse models found that ascofuranone was only curative when co-administered with a large amount (3 g/kg) of glycerol (Yabu *et al*. 1998). Despite these less than favourable initial results, the dosage of ascofuranone was improved to once again render it a promising clinical drug candidate. Yabu et al (Yabu *et al.* 2003) trialled the optimal dosage to cure *T. b. brucei* mice without glycerol and determined that 100 mg/kg intraperitoneally for 4 days and 400 mg/kg orally for 8 days completely cleared an infection, with a 50% lethal dose (LD_{50}) of >1.2 g/kg over 8 days. This study also provided evidence of the effects of ascofuranone treatment on TAO, finding that ascofuranone decreased TAO activity by 30% and increased the level of TAO expression within the cells (Yabu *et al.* 2003).

Ascofuranone was also shown to inhibit the TAO of *T. vivax,* which causes animal trypanosomiasis (Nagana) in cattle. The *T. vivax* TAO has 76% identical amino acid residues to *T. brucei* TAO (Suzuki *et al.* 2004) and the recombinant protein was shown to be three-fold more sensitive to ascofuranone. Subsequent *in vivo* testing of ascofuranone in *T. vivax* infected mice found that a single intramuscular dose of

50 mg/kg ascofuranone without glycerol was sufficient to clear an infection, which could be reduced still further to 6 mg/kg over 4 days whilst retaining 100% cure rate within 48 hours. The high efficacy of ascofuranone against *T. vivax* may make this compound a suitable drug for use against animal trypanosomiasis.

Kinetic analysis of ascofuranone inhibition of rTAO indicated a competitive mechanism of inhibition against ubiquinol (Nihei *et al.* 2003). Recent studies of ascofuranone have revealed the mechanism of inhibition, interaction with TAO and the pharmacophore responsible for the inhibitory activity of ascofuranone (Saimoto *et al*. 2013). The length of the linker chain between the aromatic ring and furanone ring was shown to be important for its inhibitory activity, where the potency of inhibitor with a propyl linker was a thousand-fold lower compared to nonyl and decyl linkers. This is likely due to the interactions between the prenyl tail and membrane lipid bilayers, where hydrophobicity of the inhibitor is influenced by the length of the prenyl tail, which is important to access the membrane-associated TAO (Mogi *et al*. 2009; Saimoto *et al.* 2013). Attempts to improve the potency and selectivity of ascofuranone-like analogs have been reported, such as the prenylphenol LL-Z1272 series by Mogi et al (Mogi *et al.* 2009) (Figure 6), although no results from *in vivo* testing have been reported to date.

Aurachin D

Recently the natural product Aurachin D (Figure 6), a ubiquinol oxidase inhibitor isolated from the bacterium *Stigmatella aurantiaca* strain Sg a15, was shown to have inhibitory activity against *T. b. gambiense* (Li *et al*. 2013). Aurachin D is a mimic of ubiquinol, with a quinolone core and prenyl chain. Li et al (Li *et al.* 2013) found that Aurachin D inhibited *T. b. gambiense* with an IC_{50} of 1 μM, with a selectivity index

greater than 35. Various analogs of Aurachin D were synthesized and tested for trypanocidal activity, but none were improved compared to the natural product and hence the compound has not been taken forward into animal models.

CONCLUSIONS AND FUTURE PERSPECTIVES

Although drugs against TAO have been studied for over 40 years, there are still no drug candidates approaching clinical trials. The search for an effective TAO inhibitor has been hampered until recently by the difficulty in obtaining a crystal structure of the relatively unstable purified protein, and the historical conflicting reports on whether inhibition of TAO alone is sufficient to kill *T. brucei in vivo.* However, recent evidence renews the idea of TAO as a valid drug target. Although there are few inhibitors of TAO reported in the literature, it is hoped that the publication of the crystal structure of TAO will significantly improve the design of novel, potent inhibitors against the enzyme. Further work is also still required to confirm the mechanism of electron transfer by TAO and that ubiquinol is the true native co-factor.

Acknowledgements

We would like to thank the rest of the members of the Gordon Florence and Terry Smith research groups.

Financial Support

This work was supported by the Leverhulme Trust (Grant number RL-2012-025)

References

Ajayi, W., Chaudhuri, M. and Hill, G.C. 2002. “Site-Directed Mutagenesis Reveals the Essentiality of the Conserved Residues in the Putative Diiron Active Site of the Trypanosome Alternative Oxidase.” *Journal of Biological Chemistry* 277 (10): 8187–93. doi:10.1074/jbc.M111477200.

Andersson, M.E. and Nordlund, P. 1999. “A Revised Model of the Active Site of Alternative Oxidase.” *FEBS Letters* 449: 17–22. doi:S0014-5793(99)00376-2 [pii].

Baker, N., de Koning, H.P., Mäser, P. and Horn, D. 2013. “Drug Resistance in African Trypanosomiasis: The Melarsoprol and Pentamidine Story.” *Trends in Parasitology* 29 (3): 110–18. doi:10.1016/j.pt.2012.12.005.

Barrett, M.P., and Croft, S.L. 2012. “Management of Trypanosomiasis and Leishmaniasis.” *British Medical Bulletin* 104 (1): 175–96. doi:10.1093/bmb/lds031.

Berthold, D.A., Andersson, M.E. and Nordlund, P. 2000. “New Insight into the Structure and Function of the Alternative Oxidase.” *Biochimica et Biophysica Acta* 1460 (2–3): 241–54. doi:10.1016/S0005-2728(00)00149-3.

Berthold, D.A., Voevodskaya, N., Stenmark, P., Gräslund, A. and Nordlund, P. 2002. “EPR Studies of the Mitochondrial Alternative Oxidase: Evidence for a Diiron Carboxylate Center.” *Journal of Biological Chemistry* 277 (46): 43608–14. doi:10.1074/jbc.M206724200.

Bringaud, F., Barrett, M.P. and Zilberstein, D. 2012. “Multiple Roles of Proline Transport and Metabolism in Trypanosomatids.” *Frontiers in Bioscience* 17: 349–74.

Chaudhuri, M., Wilfred A. and Hill, G.C.. 1998. “Biochemical and Molecular Properties of the Trypanosoma Brucei Alternative Oxidase.” *Molecular and Biochemical Parasitology* 95: 53–68.

Chaudhuri, M., Wilfred, A., Temple, S. and Hill, G.C. 1995. “Identification and Partial Purification of a Stage-Specific 33 kDa Mitochondrial Protein as the Alternative Oxidase of the Trypanosoma Brucei Brucei Bloodstream Trypomastigotes.” *The Journal of Eukaryotic Microbiology* 42 (5): 467–72.

Chaudhuri, M., and Hill, G.C. 1996. “Cloning, Sequencing and Functional Activity of the Trypanosoma Brucei Brucei Alternative Oxidase.” *Molecular and Biochemical Parasitology* 83: 125–29.

Clarkson, A.B. and Brohn, F.H. 1976. “Trypanosomiasis: An Approach to Chemotherapy by the Inhibition of Carbohydrate Catabolism.” *Science (New York, N.Y.)* 194 (4261): 204–6. doi:10.1126/science.986688.

Clarkson, A.B., Bienensb, E.J., Pollakisz, G. and Gradyll, W. 1989. “Respiration of Bloodstream Forms of the Parasite Trypanosoma Brucei Brucei Is Dependent on a Plant-like Alternative Oxidase.” *The Journal of Biological Chemistry* 264 (30): 17770–76.

Fang, J., and Beattie, D.S. 2003. “Alternative Oxidase Present in Procyclic Trypanosoma Brucei May Act to Lower the Mitochondrial Production of Superoxide.” *Archives of Biochemistry and Biophysics* 414: 294–302. doi:10.1016/S0003-9861(03)00196-6.

Fukai, Y., Amino, H., Hirawake, H., Yabu, Y., Ohta, N., Minagawa, N., Sakajo, S., Yoshimoto, A., Nagai, K., Takamiya, S., Kojima, S. and Kita, K. 1999. “Functional Expression of the Ascofuranone-Sensitive Trypanosoma Brucei Brucei Alternative Oxidase in the Cytoplasmic Membrane of Escherichia Coli.” *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology & Endocrinology* 124 (2): 141–48. doi:10.1016/S0742-8413(99)00040-7.

Fukai, Y., Coichi N., Keisuke K., Yoshisada Y. and Suzuki, T. 2003. “Overproduction of Highly Active Trypanosome Alternative Oxidase in Escherichia Coli Heme-Deficient Mutant.” *Parasitology International* 52: 237–41. doi:10.1016/S1383-5769.

Grady, R.W., Bienen, E.J., Dieck, H.A., Saric, M. and Clarkson, A.B. 1993. “N-N-Alkyl-3,4-Dihydroxybenzamides as Inhibitors of the Trypanosome Alternative Oxidase: Activity in Vitro and in Vivo.” *Antimicrobial Agents and Chemotherapy* 37 (5): 1082–85. doi:10.1128/AAC.37.5.1082.

Grant, P.T. and Sargent, J.R. 1960. “Properties of L-Alpha-Glycerophosphate Oxidase and Its Role in the Respiration of Trypanosoma Rhodesiense.” *The Biochemical Journal* 76: 229–37.

Helfert, S., Estévez, A.M., Bakker, B., Michels, P. and Clayton, C. 2001. “Roles of Triosephosphate Isomerase and Aerobic Metabolism in Trypanosoma Brucei.” *The Biochemical Journal* 357 (Pt 1): 117–25. doi:10.1042/0264-6021:3570117.

Kido, Y., Shiba, T., Inaoka, D.K., Sakamoto, K., Nara, T., Aoki, T., Honma, T., Tanaka, A., Inoue, M., Matsuoka, S., Moore, A., Harada, S. and Kita, K. 2010. “Crystallization and Preliminary Crystallographic Analysis of Cyanide-Insensitive Alternative Oxidase from Trypanosoma Brucei Brucei.” *Acta Crystallographica. Section F, Structural Biology and Crystallization Communications* 66 (Pt 3). International Union of Crystallography: 275–78. doi:10.1107/S1744309109054062.

Kuepfer, I., Schmid, C., Allan, M., Edielu, A., Haary, E.P., Kakembo, A., Kibona, S., Blum, J. and Burri, C. 2012. “Safety and Efficacy of the 10-Day Melarsoprol Schedule for the Treatment of Second Stage Rhodesiense Sleeping Sickness.” *PLoS Neglected Tropical Diseases* 6 (8). doi:10.1371/journal.pntd.0001695.

Li, X.W., Herrmann, J., Zang, Y., Grellier, P., Prado, S., Muller, R. and Nay, B. 2013. “Synthesis and Biological Activities of the Respirator Chain Inhibitor Aurachin D and New Ring versus Chain Analogues.” *Beilstein Journal of Organic Chemistry* 9 (Figure 1): 1551–58. doi:10.3762/bjoc.9.176.

Lüscher, A., de Koning, H.P. and Mäser, P. 2007. “Chemotherapeutic Strategies against Trypanosoma Brucei: Drug Targets vs. Drug Targeting.” *Current Pharmaceutical Design* 13 (April): 555–67. doi:10.2174/138161207780162809.

Marechal, A., Kido, Y., Kita, K., Moore, A.L. and Rich, P.R. 2009. “Three Redox States of Trypanosoma Brucei Alternative Oxidase Identified by Infrared Spectroscopy and Electrochemistry.” *The Journal of Biological Chemistry* 284 (46): 31827–33. doi:10.1074/jbc.M109.059980.

Minagawa, N., Yabu, Y., Kita, K., Nagai, K., Ohta, N., Meguro, K., Sakajo, S.and Yoshimoto, A. 1997. “An Antibiotic, Ascofuranone, Specifically Inhibits Respiration and in Vitro Growth of Long Slender Bloodstream Forms of Trypanosoma Brucei Brucei.” *Molecular and Biochemical Parasitology* 81 (2): 127–36. doi:10.1016/0166-6851(96)02665-5.

Mogi, T., Ui, H., Shiomi, K., Omura, S., Miyoshi, H. and Kita, K. 2009. “Antibiotics LL-Z1272 Identified as Novel Inhibitors Discriminating Bacterial and Mitochondrial Quinol Oxidases.” *Biochimica et Biophysica Acta - Bioenergetics* 1787 (2): 129–33. doi:10.1016/j.bbabio.2008.11.016.

Moore, A.L., Carré, J.E., Affourtit, C., Albury, M.S., Crichton, P.G., Kita, K. and Heathcote, P. 2008. “Compelling EPR Evidence That the Alternative Oxidase Is a Diiron Carboxylate Protein.” Article. *Biochimica et Biophysica Acta* 1777 (4): 327—330. doi:10.1016/j.bbabio.2008.01.004.

Moore, A.L., Shiba, T.,Young, L., Harada, S., Kita, K. and Ito,K. 2013. “Unraveling the Heater: New Insights into the Structure of the Alternative Oxidase.” *Annual Review of Plant Biology* 64 (January): 637–63. doi:10.1146/annurev-arplant-042811-

105432.

Nakamura, K., Fujioka, S., Fukumoto, S., Inoue, N., Sakamoto, K., Hirata, H., Kido, Y., Yabu, Y., Suzuki, T., Watanabe, Y.I., Saimoto, H., Akiyama, H. and Kita, K. 2010. “Trypanosome Alternative Oxidase, a Potential Therapeutic Target for Sleeping Sickness, Is Conserved among Trypanosoma Brucei Subspecies.” *Parasitology International* 59 (4). Elsevier Ireland Ltd: 560–64. doi:10.1016/j.parint.2010.07.006.

Nihei, C., Fukai, Y., Kawai, K., Osanai, A., Yabu, Y., Suzuki, T., Ohta, N., Minagawa, N., Nagai, K. and Kita, K. 2003. “Purification of Active Recombinant Trypanosome Alternative Oxidase.” *FEBS Letters* 538 (1–3): 35–40. doi:10.1016/S0014-5793(03)00120-0.

Nihei, C., Fukai, Y. and Kita, K. 2002. “Trypanosome Alternative Oxidase as a Target of Chemotherapy.” *Biochimica et Biophysica Acta - Molecular Basis of Disease* 1587 (2–3): 234–39. doi:10.1016/S0925-4439(02)00086-8.

Opperdoes, F.R., Borst, P. and Fonck, K. 1976. “The Potential Use of Inhibitors of Glycerol-3-Phosphate Oxidase for Chemotherapy of African Trypanosomiasis.” *FEBS Letters* 62 (2): 169–72. doi:10.1016/0014-5793(76)80045-2.

Ott, R., Chibale, K., Anderson, S., Chipeleme, A., Chaudhuri, M., Guerrah, A., Colowick, N. and Hill, G.C. 2006. “Novel Inhibitors of the Trypanosome Alternative Oxidase Inhibit Trypanosoma Brucei Brucei Growth and Respiration.” *Acta Tropica*

100 (3): 172–84. doi:10.1016/j.actatropica.2006.10.005.

Pollakis, G., Grady, R.W., Dieck, H.A. and Clarkson, A.B. 1995. “Competition between Inhibitors of the Trypanosome Alternative Oxidase (TAO) and Reduced Coenzyme Q9.” *Biochemical Pharmacology* 50 (8): 1207–10. doi:10.1016/0006-2952(95)00259-3.

Saimoto, H., Kido, Y., Haga, Y., Sakamoto, K. and Kita, K. 2013. “Pharmacophore Identification of Ascofuranone, Potent Inhibitor of Cyanide-Insensitive Alternative Oxidase of Trypanosoma Brucei.” *Journal of Biochemistry* 153 (3): 267–73. doi:10.1093/jb/mvs135.

Schonbaum, G.R., Bonner, W.D., Storey, B.T. and Bahr, J.T. 1971. “Specific Inhibition of the Cyanide-Insensitive Respiratory Pathway in Plant Mitochondria by Hydroxamic Acids.” *Plant Physiology* 47 (1): 124–28. doi:10.1104/pp.47.1.124.

Shiba, T., Kido, Y., Sakamoto, K., Ken, D., Tsuge, C. and Tatsumi, R. 2013. “Structure of the Trypanosome Cyanide-Insensitive Alternative Oxidase.” *Proceedings of the National Academy of Sciences* 110 (12): 4580–85. doi:10.1073/pnas.1218386110/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1218386110.

Simarro, P.P., Cecchi, G., Franco, J.R., Paone, M., Diarra, A., Ruiz-Postigo, J.A., Fèvre, E.M., Mattioli, R.C. and Jannin, J.G. 2012. “Estimating and Mapping the Population at Risk of Sleeping Sickness.” *PLoS Neglected Tropical Diseases* 6 (10):

545 e1859. doi:10.1371/journal.pntd.0001859.
546
547 **Suzuki, T., Nihei, C.I.,Yabu, Y., Hashimoto, T., Suzuki, M., Yoshida, A., Nagai,**
548 **K., Hosokawa, T., Minagawa, N., Suzuki, S., Kita, K. and Ohta, N.** 2004.
549 "Molecular Cloning and Characterization of Trypanosoma Vivax Alternative Oxidase
550 (AOX) Gene, a Target of the Trypanocide Ascofuranone." *Parasitology International*
551 53 (3): 235–45. doi:10.1016/j.parint.2004.02.001.
552
553 **Tsuda, A., Witola, W.H., Konnai, S., Ohashi, K. and Onuma, M.** 2006. "The Effect
554 of TAO Expression on PCD-like Phenomenon Development and Drug Resistance in
555 Trypanosoma Brucei." *Parasitology International* 55 (2): 135–42.
556 doi:10.1016/j.parint.2006.01.001.
557
558 **Vassella, E., Probst, M., Schneider, A., Studer, E., Renggli, C.K., and Roditi, I**.
559 2004. "Expression of a Major Surface Protein of Trypanosoma Brucei Insect Forms
560 Is Controlled by the Activity of Mitochondrial Enzymes." *Molecular Biology of the Cell*
561 15 (9): 3986–93. doi:10.1091/mbc.E04-04-0341.
562
563 **Walker, R., Saha, L., Hill, G.C. and Chaudhuri, M.** 2005. "The Effect of over-
564 Expression of the Alternative Oxidase in the Procyclic Forms of Trypanosoma
565 Brucei." *Molecular and Biochemical Parasitology* 139 (2): 153–62.
566 doi:10.1016/j.molbiopara.2004.11.003.
567
568 **World Health Organization.** 2013. "Control and Surveillance of Human African
569 Trypanosomiasis." *World Health Organization Technical Report Series*, no. 984: 1–

237.

World Health Organization. 2016. “Human African Trypanosomiasis.” http://apps.who.int/gho/data/node.main.A1635?lang=en.

Yabu, Y., Minagawa, N., Kita, K., Nagai, K., Honma, M., Sakajo, S., Koide, T., Ohta, N. and Yoshimoto, A. 1998. “Oral and Intraperitoneal Treatment of Trypanosoma Brucei Brucei with a Combination of Ascofuranone and Glycerol in Mice.” *Parasitology International* 47 (2): 131–37. doi:10.1016/S1383-5769(98)00011-7.

Yabu, Y., Suzuki, T., Nihei, C.I., Minagawa, N., Hosokawa, T., Nagai, K., Kita, K. and Ohta, N. 2006. “Chemotherapeutic Efficacy of Ascofuranone in Trypanosoma Vivax-Infected Mice without Glycerol.” *Parasitology International* 55 (1): 39–43. doi:10.1016/j.parint.2005.09.003.

Yabu, Y., Yoshida, A., Suzuki, T., Nihei, C.I., Kawai, K., Minagawa, N., Hosokawa, T., Nagai, K., Kita, K. and Ohta, N. 2003. “The Efficacy of Ascofuranone in a Consecutive Treatment on Trypanosoma Brucei Brucei in Mice.” *Parasitology International* 52 (2): 155–64.

Zoltner, M., Horn, D., de Koning, H.P., and Field, M.C. 2016. “Exploiting the Achilles’ Heel of Membrane Trafficking in Trypanosomes.” *Current Opinion in Microbiology* 34. Elsevier Ltd: 97–103. doi:10.1016/j.mib.2016.08.005.

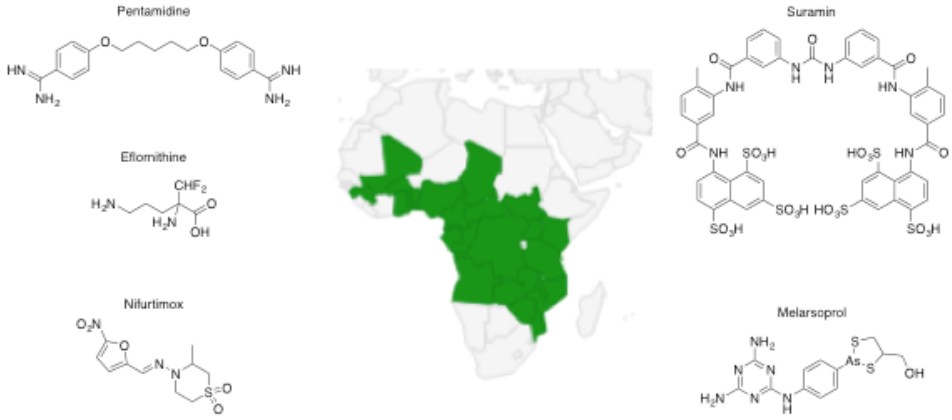

Figure 1. Distribution of countries endemic for Trypanosoma brucei *according to WHO and the currently used clinical drugs*

595

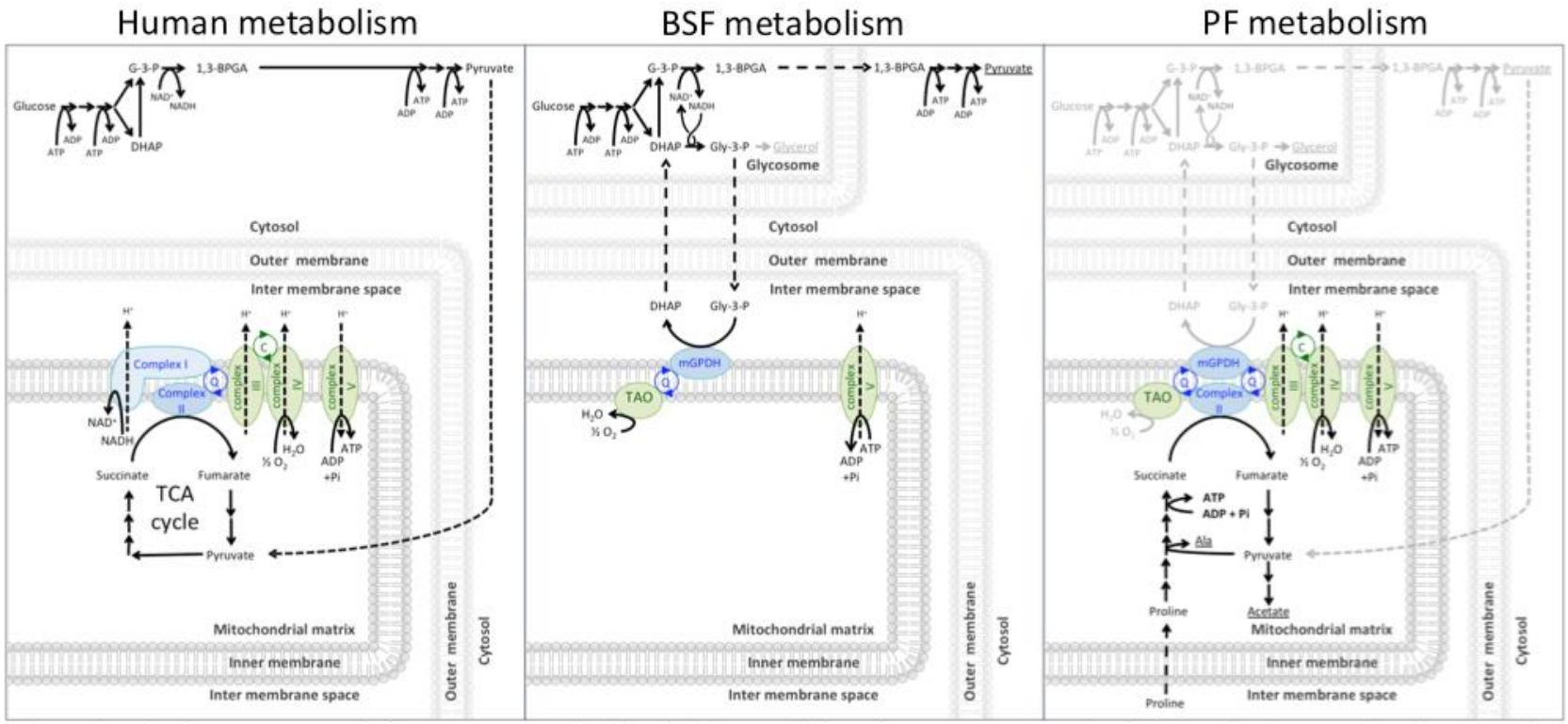

Figure 2. Carbon source metabolism. Human cells glycolyticaly metabolise glucose to pyruvate in the cytosol. Pyruvate is taken into the mitochondrial matrix where it is further completely metabolised to CO2 and water through the TCA cycle and electron transport chain (shown in green). Entry points to the electron transport chain are shown in blue. The malate-aspartate shuttle (not shown) maintains cytosolic NAD(H) redox.

BSF T. brucei metabolise glucose to 1,3-BPGA in the glycosome and 1,3-BPGA to pyruvate in the cytosol. A high rate of glycolysis means that sufficient ATP is produced through this rout alone and the parasite can secrete the pyruvate produced as waste rather than spend energy consuming it further. The GPO system (mGPDH and TAO) is required to maintain glycolytic NAD(H) redox. If the GPO system is inhibited BSF T. brucei will convert Gly-3-P to the secreted end product glycerol to maintain glycolytic NAD(H) redox.

PF T. brucei are able to metabolise glucose, however, in the insect midgut glucose is so low in abundance that the main carbon source is proline. Proline is converted to acetate and alanine in a TCA-like, non-cyclical fashion. ATP is generated through oxidative phosphorylation and TAO, present at low levels, is hardly used.

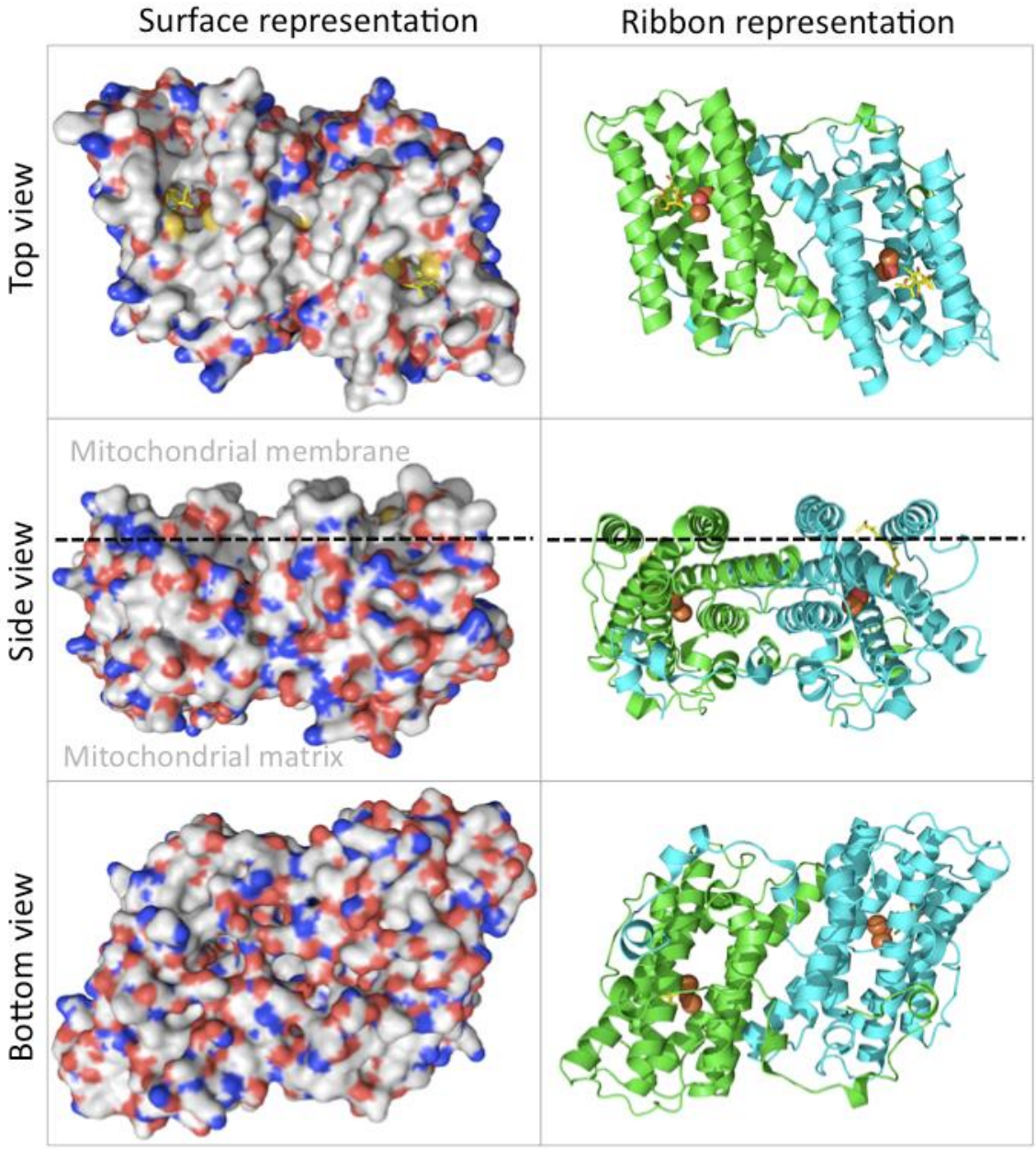

Figure 3. The TAO dimer in complex with diiron/hydroxo core (shown as spheres) and inhibitor AF2779OH (shown in yellow carbon stick). For surface representation, hydrophobic areas are grey and hydrophilic areas are blue/red. The upper face of the dimer is highly hydrophobic allowing TAO to burry itself within a single layer of the inner mitochondrial membrane. The approximate position of the membrane/matrix interface is represented by a dashed line. The diiron catalytic core is burried deep within the protein structure and a channel from the membrane to the core allows access of ubiquinol substrate (or analogous inhibitor AF2779OH).

598

599

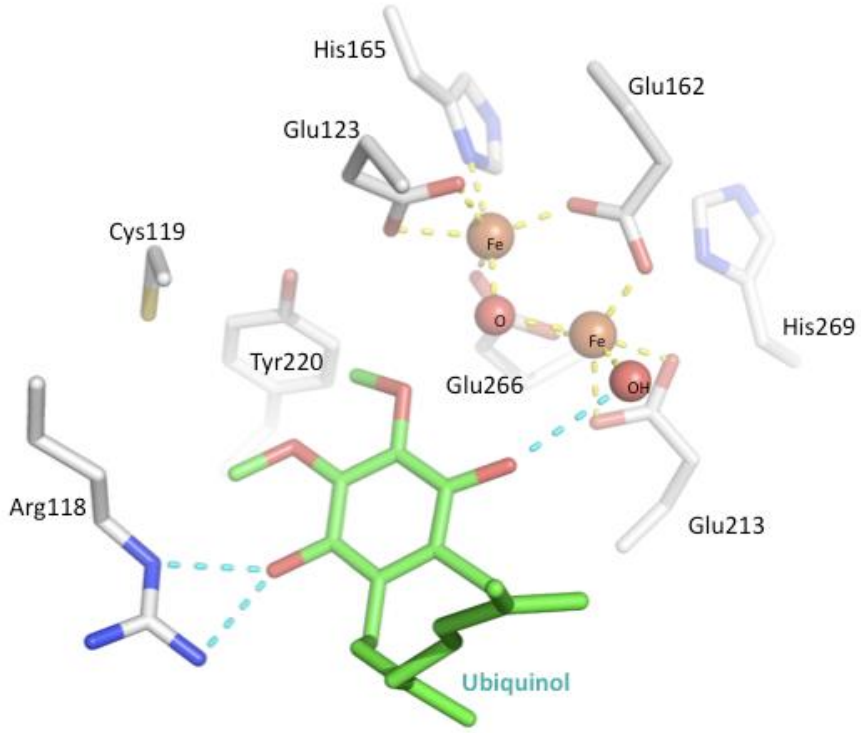

Figure 4. The TAO active site with ubiquinol (green) superposed in the place of inhibitor AF2779OH. The diiron core is in theFe(III/III) oxodiiron state (see mechanism part in the main body text), coordinated by four glutamates, two histidines, an oxygen and a hydroxyl (yellow dashed lines).

For the first ubiquinol oxidation, the coordinated OH would abstract a hydrogen atom (proton plus electron) from ubiquinol (blue dashed line) and leave as water, reducing the core to Fe(II/III) and oxidising ubiquinol to semiubiquinol. The oxygen bridge will take a hydrogen from Tyr220 to form a hydroxo bridge and the resulting tyrosyl radical will take a hydrogen from semiubiquinol either directly or via Cys119 and return to its native state, leaving the core in a reduced diferrous Fe(II/II) state bridged by a sinle hydroxyl. With the reaction complete, ubiquinone will leave allowing molecular oxygen and a second ubiquinol to enter.

600

601

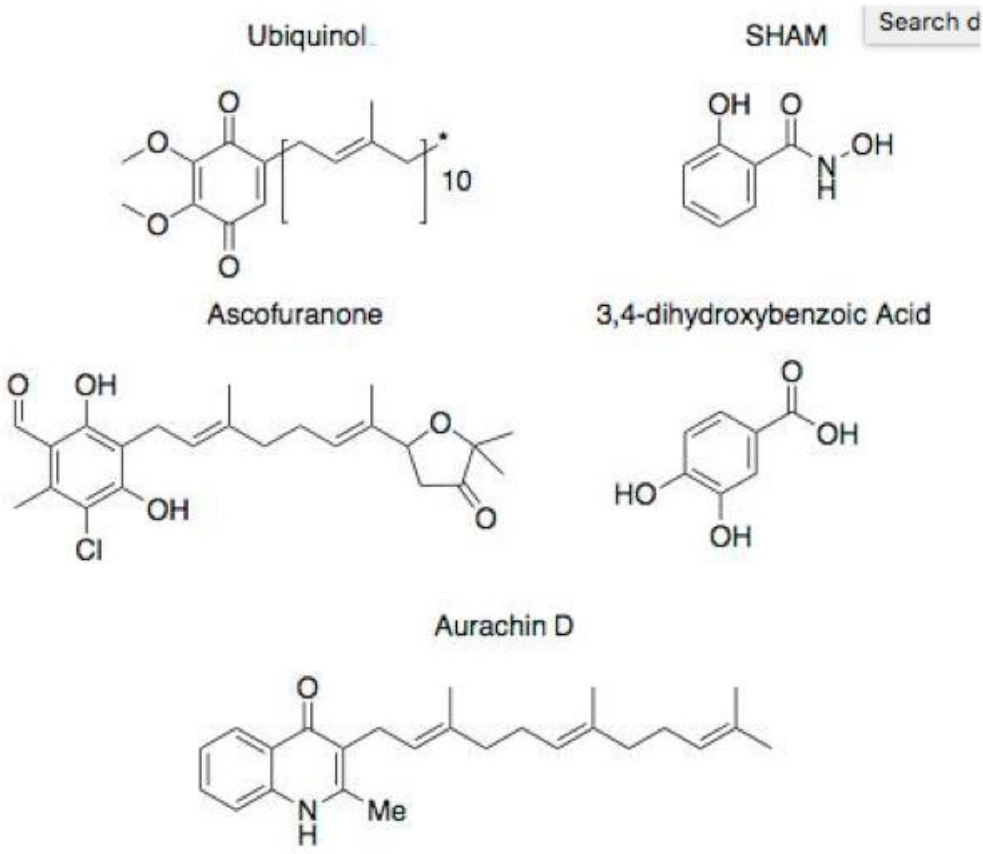

Figure 5. Chemical structures of TAO substrate ubiquinol and the TAO inhibitors salicylhydroxamic acid (SHAM), ascofuranone, 3,4-dihydroxybenzoic acid and Aurachin D.

602

603

604